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Functional binding of the "TATA" box binding component of transcription factor TFIID to the −30 region of TATA-less promoters

(human dihydrofolate reductase promoter/RNA polymerase II/simian virus 40 major late promoter/terminal deoxynucleotidyltransferase promoter/transcription)

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ABSTRACT **Many viral and cellular promoters transcribed in higher eukaryotes by RNA polymerase II lack obvious A+T-rich sequences, called "TATA" boxes, that bind the transcription factor TFIID. One such TATA-less promoter, the simian virus 40 major late promoter, contains a genetically important sequence element 30 base pairs upstream of its transcription initiation site that has no obvious sequence similarity to a TATA box. We show here that the cloned human TATA box-binding protein, hTFIIDτ, functionally binds to this upstream sequence element, although with an affinity one-sixth of that to which it binds the TATA box of the adenovirus type 2 major late promoter. Analysis of point mutations in the −30 element of the simian virus 40 major late promoter shows that the affinity of binding correlates with the efficiency of transcription from this promoter. Furthermore, this element has genetic properties similar to those of a TATA box. (*i*) It directs RNA polymerase II to initiate transcription ≈30 base pairs downstream of its location, and (*ii*) inactivation of this element results in increased heterogeneity in the sites of transcription initiation. All of five other TATA-less promoters tested were found to contain a sequence ≈30 base pairs upstream of their major transcription initiation sites to which hTFIIDτ binds. We conclude that many, if not all, TATA-less promoters differ from TATA box-containing promoters simply in the affinity of their −30 regions for binding of TFIID, with functional binding of TFIID supported in part by other nearby sequence elements of the promoter.**

The mechanism of transcription initiation by RNA polymerase II in higher eukaryotes has been best characterized for promoters such as the adenovirus 2 major late promoter (Ad-MLP) that have an A+T-rich sequence, often resembling "TATAA," located ≈30 base pairs (bp) upstream of the initiation site (1). An early step in the formation of transcription initiation complexes on these promoters is the binding of the cellular factor TFIID to their "TATA" boxes (2). These TATA box sequence elements, in conjunction with sequences ≈30 bp away at the site of initiation (3), determine the precise location of transcription initiation by RNA polymerase II.

Despite the apparent central importance of TFIID in transcription initiation by RNA polymerase II, numerous promoters transcribed by this polymerase have been found that lack a TATA box ≈30 bp upstream of their initiation site (for review, see ref. 4). Among these TATA-less promoters are the simian virus 40 (SV40) MLP (for review, see ref. 5) and promoters for murine terminal deoxynucleotidyltransferase (TdT) (6), human dihydrofolate reductase (7), interferon regulatory factor (8), and mouse ribosomal protein gene *rpL32* (9). Although these promoters exhibit various degrees of heterogeneity in their sites of transcription initiation, they initiate predominantly at one site despite their lack of a TATA box.

The SV40 late promoter is one of the best-characterized TATA-less promoters. Although transcription initiates at numerous sites (ref. 10 and references therein), the major late initiation site at nucleotide (nt) 325 accounts for 70–80% of the steady-state viral RNA accumulated by late times after infection of monkey and human cells (11). Three sequence elements, located in the regions −30, +1, and +30 relative to the major start site, are required for significant transcription initiation (5). It has been shown that the −30 region element can be replaced by a sequence more closely resembling a TATA box (12, 13). We show that the cloned human TATA box binding component of TFIID, referred to as hTFIIDτ (14), directly binds the SV40-MLP −30 region element in a manner functionally analogous to the way it binds the TATA box of TATA box-driven promoters, albeit with a somewhat lower affinity. Each of several other TATA-less promoters tested also contains a sequence ≈30 bp upstream of its major transcription initiation site that binds hTFIIDτ. We conclude that, in many cases, the difference between TATA box-containing and TATA-less promoters with respect to upstream binding of TFIID is a quantitative difference in the affinity with which TFIID by itself binds the −30 region.

MATERIALS AND METHODS

DNAs. All plasmid DNAs were constructed by standard recombinant DNA techniques (15). Plasmid pSVS contains wild-type (WT) SV40 830 inserted at its *Eco*RI site into a pBR322-derived vector (16). Plasmid pSV1770(sub294) is a derivative of pSVS in which SV40 nt 294–298, inclusive, containing the TFIID binding site, has been replaced with 5′-dCCTCGAGG-3′ (17). Plasmid pSV1790 is a derivative of pSVS made by E. Murray of our laboratory (personal communication), in which SV40 nt 319–336, inclusive, has been replaced with the sequence 5′-dCTGGGCAGGTCTCGAGACCTGCCCAG-3′. Plasmid pSV1791(dup309), containing the 10-bp direct repeat of SV40 nt 309–318, inclusive, was constructed by hybridization and ligation of the synthetic oligonucleotides 5′-TTCATCCTCTTTCAGAGGTTATTTCAGGCCAT-3′ and 5′-CACCATGGCCTGAAATAACCTCTGAAAGAGGA-3′ into *Bsp*MI-cut pSV1790. Plasmid pSV1792(dup320), containing the 10-bp direct repeat of SV40 nt 320–329, inclusive, was constructed by hybridization and ligation of the synthetic oligonucleotides 5′-

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Abbreviations: SV40, simian virus 40; MLP, major late promoter; Ad, adenovirus; TdT, terminal deoxynucleotidyltransferase; WT, wild type; nt, nucleotide(s).

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TTCAGAGGTTATTTCAGGTTATTTCAGGCCAT-3′ and 5′-CACCATGGCCTGAAATAACCTGAAATAACCTC-3′ into *Bsp*MI-cut pSV1790. Plasmid pSV1793(ins319), inserting CAGT repeats at SV40 nt 319, was constructed by hybridization and ligation of the synthetic oligonucleotides 5′-TTCAGTCAGTCAGTGAGGTTATTTCAGGCCAT-3′ and 5′-CACCATGGCCTGAAATAACCTCACTGACTGAC-3′ into *Bsp*MI-cut pSV1790.

Competition Gel-Mobility-Shift Assays. Competition gel-mobility-shift assays were performed as described (18) with the 5′-end-labeled oligonucleotide 5′-AAGGGGGGCTATAAAAGGGGGTGG-3′ (spanning the TATA box of the Ad-MLP) hybridized to its complementary oligonucleotide serving as probe. Each lane in Figs. 1 and 3 contained 10 ng of 90% pure bacterially expressed hTFIIDτ (ref. 18; Promega) and an equimolar amount of probe unless otherwise indicated. Lanes lacking hTFIIDτ contained equivalent volumes of a protein sample prepared identically to hTFIIDτ, except from bacteria carrying an hTFIIDτ^- variant plasmid (Promega). The molar ratios and sequences of the double-stranded oligonucleotides used as competitor DNA are indicated in the figures. Relative affinities were calculated by taking the inverse of the ratio of moles of competitor required for a 50% reduction of shifted radioactivity as determined by laser densitometric analysis of autoradiograms. Competition experiments were repeated at least three times using two preparations of hTFIIDτ; each gave similar results.

Transcription Assays. Cell-free transcription assays were performed essentially as described by Dignam *et al.* (19) with nuclear extract made from HeLa cells, circular plasmid DNAs as templates (250 ng of plasmid DNA per 25 μl of reaction mixture), and the addition of 80 mM potassium glutamate. *In vivo* transcription assays were performed by transfection of CV-1PD cells and purification of whole-cell RNA as described by Good *et al.* (11). The RNA products were analyzed by the primer-extension technique (17) with a synthetic oligonucleotide of SV40 nt 394–369 serving as primer.

RESULTS

hTFIIDτ Binds the −30 Region of TATA-Less Promoters. A DNase I protection assay was used to test whether the SV40 MLP and TdT promoter contain sequences that specifically bind the TATA box-binding component of human TFIID, hTFIIDτ. Surprisingly, by using a slightly higher protein-to-DNA ratio than needed to protect the TATA box region of the Ad-MLP from cleavage, we observed protection by this highly purified *Escherichia coli*-synthesized protein at the −30 through −15 region of the SV40 MLP and the −40 through −22 region of the TdT promoter (data not shown). Competition gel-shift assays (see below) confirmed that hTFIIDτ can bind to the −30 regions of certain promoters lacking apparent TATA box motifs.

Correlation of hTFIIDτ Binding with Transcription. If binding of TFIID to the −30 region is physiologically important, mutations in this region affecting transcription should also affect binding of TFIID. Point mutations in the −30 region of the SV40-MLP can alter the transcriptional activity of this promoter (12, 13): some mutations decrease transcriptional activity (e.g., pSVsKA1 in Fig. 1*B*) and others increase it (e.g., pSVsKC2 in Fig. 1*B*). The relative abilities of 20-bp double-stranded oligonucleotides containing the −30 regions of WT SV40-MLP and the two point mutants pSVsKA1 and pSVsKC2 (Fig. 1*B*) to compete for binding of hTFIIDτ to a 24-bp double-stranded oligonucleotide containing the TATA box of the Ad-MLP were measured by competition gel-mobility-shift assays (Fig. 1*A*). The SV40 WT oligonucleotide competed for binding (Fig. 1*A*, lanes 3–6), albeit with an affinity one-sixth of that to which it binds the Ad-MLP probe

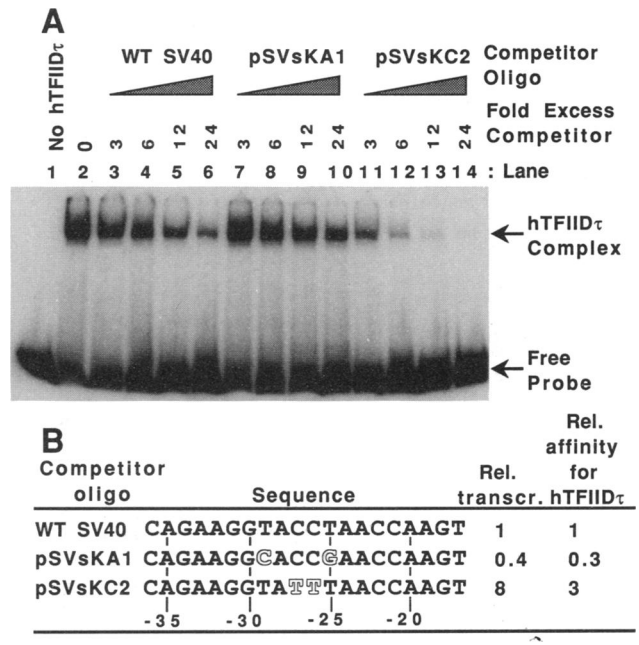

FIG. 1. hTFIIDτ binding to the −30 region correlates with transcription of the SV40-MLP. (*A*) SV40 −30 region mutants differ in their ability to bind hTFIIDτ. An autoradiogram of competition gel-mobility-shift assays is shown. Each reaction mixture contained 4 ng of 5′-end-labeled 24-bp Ad-MLP TATA box probe DNA, 10 ng of TFIIDτ, and the fold molar excess of the 20-bp competitor oligonucleotide indicated, the sequence of which is shown in *B*. (*B*) Summary of data on transcription vs. hTFIIDτ binding of WT and two −30 region mutants of SV40. The relative transcriptional activities (Rel. transcr.) in a HeLa cell nuclear extract system were taken from Nandi *et al.* (13).

(see below). The pSVsKC2 oligonucleotide competed better for binding of hTFIIDτ than did the wild-type oligonucleotide (Fig. 1*A*, lanes 11–14), whereas the pSVsKA1 oligonucleotide competed less well (Fig. 1*A*, lanes 7–10) (see Fig. 1*B* for summary). Therefore, the efficiency of transcription initiation at the SV40-MLP correlates with the affinity of binding of hTFIIDτ to its −30 region.

Genetic Properties of the SV40-MLP TFIID Binding Site. If binding of hTFIIDτ to the −30 region of the SV40-MLP has functional significance, one would also predict that insertion of base pairs between this TFIID binding site and the major initiation site at nt 325 should result in a shift in the initiation site such that it remains ≈30 bp downstream of the hTFIIDτ binding site (1). To test this prediction, we constructed pSV1791(dup309), a variant of pSVS(WT) containing the 10-bp direct repeat of SV40 nt 309–318, inclusive (Fig. 2*C*). Analysis of the 5′ ends of the transcripts synthesized from this mutated promoter in transfected monkey cells showed that 50% of transcription initiations are no longer at nt 325; rather, they have shifted upstream to two locations, nt 318 and 321, 30–32 bp from the TFIID binding site (Fig. 2*B*, lanes 4 vs. 2). In a cell-free transcription system, this mutation results in greater heterogeneity of initiation than *in vivo*, with nt 321 becoming the major start site and the amount of transcription initiating at nt 325 being reduced to one-sixth of that observed with the WT promoter (Fig. 2*A*, lanes 4 vs. 2). Since the exact locations of these start sites are probably influenced by the preference of RNA polymerase II to initiate at certain sequences (1), two additional constructs, pSV1793(ins319), containing an insertion of three CAGT repeats, and pSV1792(dup320), containing a 10-bp direct repeat of the SV40-MLP initiation site (Fig. 2*C*), were tested. Transcripts initiated *in vivo* with ins319 at nt 316 at nearly the same intensity as they did with dup309 at nt 318 (Fig. 2*B*, lane

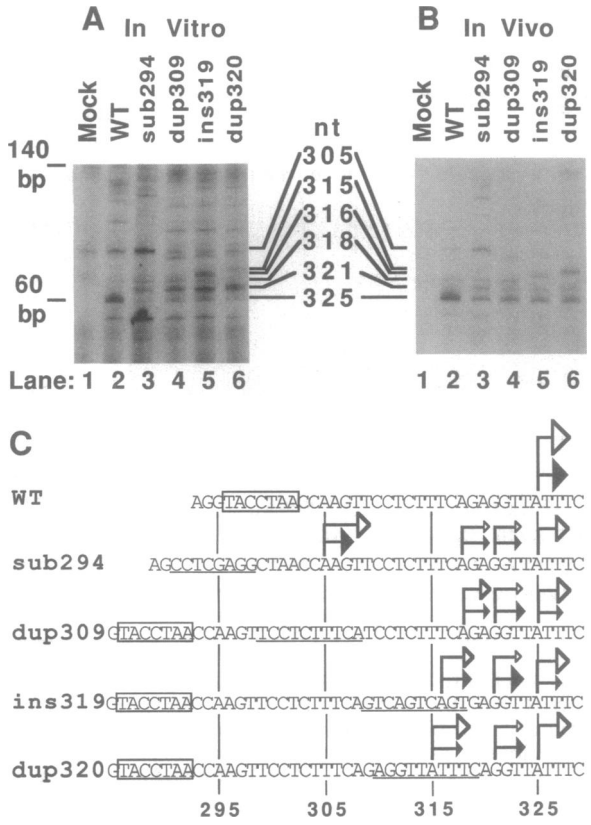

FIG. 2. SV40-MLP −30 region positions the site of transcription initiation. (*A*) Autoradiogram of primer-extension analysis of SV40 late RNAs synthesized in a cell-free transcription system from the following sequences. Lanes: 2, pSVS(WT), wild-type SV40; 3, pSV1770(sub294), a −30 region substitution mutant; 4, pSV1791(dup309), a mutant with a 10-bp duplication between the TFIID binding site and the major late initiation site; 5, pSV1793(ins319), a mutant with three CAGT repeats inserted between the TFIID binding site and the major late initiation site; 6, pSV1792(dup320), a mutant containing a 10-bp duplication of the major late initiation site. Positions of DNA size markers are indicated on the left. The numbers on the right indicate the nucleotide residue numbers of the 5′ ends of SV40 late RNAs, with the major late initiation site at SV40 nt 325. (*B*) Similar analysis of these SV40 mutants transcribed in transfected monkey cells. (*C*) Diagram summarizing the locations of the 5′ ends of the SV40-MLP mRNAs synthesized *in vitro* (solid arrows) and *in vivo* (open arrows) relative to the location of the hTFIIDτ binding site. Underlined bases represent sequences not present in the WT SV40-MLP. The boxed sequences correspond to the TATAAA motif of the Ad-MLP TATA box. The sizes of the arrows indicate the relative amounts of RNA initiating at particular nucleotides near nt 325; they were determined by quantitative densitometric analysis of autoradiograms similar to those shown in *A* and *B*.

5). With ins319, 5-fold more transcripts initiated *in vitro* at nt 316 and 321 combined than at nt 325 (Fig. 2*A*, lane 5). With dup320, approximately half of the start sites were shifted *in vivo* from nt 325 to nt 315 (Fig. 2*B*, lane 6); *in vitro*, most were shifted upstream to nt 321 (Fig. 2*A*, lane 6). These shifts in the start sites are most likely due to the −30 sequence element, since (*i*) linker-scanning analysis shows that no other upstream element is required for initiation of transcription at nt 325 (5) and (*ii*) a 10-bp insertion between the major late start site and the downstream sequence element does not shift initiation of transcription in the downstream direction (R.J.K., unpublished data). In addition, these results demonstrate that the sequence at the start site influences the precise location and strength of initiation.

Another well-known property of physiologically important TFIID binding sites is that inactivation results in transcription initiating at numerous sites (1, 3). As an additional test of the functional significance of the binding of hTFIIDτ to the −30 region of the SV40-MLP, transcripts from pSV1770(sub294), a plasmid containing a substitution mutation in the TFIID binding site (Fig. 2*C*), were synthesized *in vitro* (Fig. 2*A*, lane 3) and *in vivo* (Fig. 2*B*, lane 3). This mutation resulted in both a 3- to 6-fold reduction in transcription initiation at nt 325 and an increase in initiation at numerous other sites throughout the SV40 late promoter region. Competition gel-shift analysis showed that the affinity of hTFIIDτ for this mutated sequence is one-sixth of its affinity for the WT SV40 −30 region binding site (data not shown). These data provide evidence that this TFIID binding site on the SV40-MLP functions in a fashion analogous to a TATA box.

hTFIIDτ Binding to −30 Regions of Other TATA-Less Promoters. To determine whether other TATA-less promoters might also possess binding sites for hTFIIDτ ≈30 bp upstream of their major initiation sites, competition gel-mobility-shift assays were performed as in Fig. 1 with a variety of 20-bp oligonucleotide competitor DNAs containing the −30 regions of other TATA-less promoters (Figs. 3 and 4). All of the −30 region oligonucleotides of the promoters tested, including the 70% G+C-rich rpL32 oligonucleotide, competed as well as or better than the functional TFIID binding site of the SV40-MLP (Fig. 3; summarized in Fig. 4). On the other hand, a 50% A+T-rich double-stranded oligonucleotide corresponding to the sub294 mutation and the double-stranded sequence 5′-ACTCAGACTGTCAGTCTGAT-3′ competed one-sixth as well (data not shown). The negative control oligonucleotide (Fig. 3, lanes 21–23) failed to compete for binding for hTFIIDτ even at amounts of competitor as high as 4000 μg (data not shown). Thus, A+T content alone cannot account of the binding affinities of hTFIIDτ for these various sequences. Rather, all RNA polymerase II TATA-less promoters tested have a sequence in their −30 region that can bind hTFIIDτ, probably in a functional manner.

DISCUSSION

The SV40 MLP lacks a TATA box. Nevertheless, a variety of biochemical (Fig. 1) and genetic (Figs. 1 and 2) data presented here and by others (5, 12) provide strong evidence that binding of TFIID 30 bp upstream of the transcription initiation site plays a major role in transcription initiation from this promoter. Also presented is biochemical evidence that TFIID binds to the −30 region of other TATA-less promoters as well, albeit with weaker affinities than it binds to consensus TATA box sequences (Figs. 3 and 4). Previously published data (6, 9) indicated that the −30 regions of the TdT and rpL32 promoters are important for transcription initiation. Thus, we conclude that the binding of TFIID to the −30 region probably plays a central role in initiation of transcription of many TATA-less and TATA box-containing promoters.

Several models for initiation of transcription at TATA-less promoters have been proposed. One model, proposed by Pugh and Tjian (21, 22) to explain the requirement of extensively purified TFIID for *in vitro* transcription of synthetic TATA-less promoters (21–23), is that TFIID is stabilized in the initiation complex solely by interaction of "tether" proteins with other sequence-specific binding proteins present on the promoter. A second model is that sequences at the site of initiation, called initiator elements, determine the site of transcription initiation by direct and indirect interaction with components of the transcription machinery (for review, see ref. 4).

Our data provide further evidence that sequences at or near the site of initiation are important determinants of the initi-

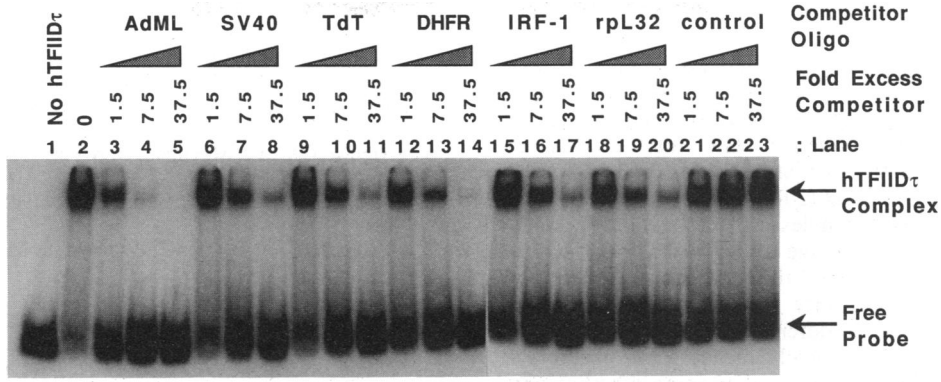

FIG. 3. The −30 regions of TATA-less promoters compete to various degrees for binding of hTFIIDτ. Competition gel-mobility-shift assays were performed as described in Fig. 1*A*. The fold excess of double-stranded competitor used is indicated above each lane. The sequences of the competitors are shown in Fig. 4. The sequence used in the control lanes was 5′-GCTGCGCCGGCTGTCACGCCAG-3′. ML, major late; DHFR, dihydrofolate reductase; IRF-1, interferon regulatory factor.

ation site. In addition, they indicate that pure hTFIIDτ can bind to diverse sequences, with direct binding of hTFIIDτ to the −30 regions of many TATA-less promoters probably also contributing to determining the initiation site. Therefore, we propose a model (Fig. 5), the "context hypothesis," in which diverse sequences can serve to bind TFIID ≈30 bp upstream of the site of initiation, thereby contributing to the formation of the initiation complex provided they are in a proper context of other sequence elements upstream, downstream, and at the site of initiation. This hypothesis can be reconciled with the tethering hypothesis, since association with the rest of the transcription machinery, possibly by associated factors (for review, see ref. 24), is required to stabilize hTFIIDτ binding to −30 region elements. The context hypothesis predicts that, although the affinity of hTFIIDτ for these sequences may be less than its affinity for consensus TATA boxes, (*i*) the function of TFIID is not qualitatively different from its function at TATA box-containing promoters and (*ii*) the affinity of hTFIIDτ for the −30 region may inversely correlate with the requirement of these flanking sequences for efficient initiation. Specifically, binding of TFIID is an early step in formation of a stable initiation complex and is a major but not exclusive determinant of the site of initiation. Evidence presented here in support of this hypothesis is as follows. (*i*) Some binding of hTFIIDτ to the −30 region of TATA-less promoters can occur in the absence of other proteins (Figs. 1 and 3). (*ii*) hTFIIDτ binding affinity correlates with the amount of initiation at the SV40-MLP (Fig. 1). (*iii*) Sequences 30 bp downstream of a TFIID binding site play a role in determining the precise site(s) and efficiency of initiation (Fig. 2). Also consistent with the context hypothesis are the findings of (*i*) Lieberman and Berk (25) that Zta, a DNA-binding protein encoded by Epstein–Barr virus, stabilizes binding of TFIID to Zta-responsive promoters and (*ii*) Zenzie-Gregory *et al.* (26) that TATA box- and initiator element-containing promoters probably initiate transcription by a similar mechanism.

It has been demonstrated that yeast TFIID can bind to nonconsensus TATA sequences (27). Singer and Struhl (28) have reported that 1% of random DNA can functionally substitute for a TATA box in the *his3* promoter of yeast. It is a possibility that some of these mutated sequences bind as-yet-undiscovered functional equivalents of hTFIIDτ with different sequence specificities. However, we consider this unlikely in light of the evidence presented here and elsewhere (21–23) supporting a role of hTFIIDτ. More consistent with the data is the hypothesis that, in a proper context of flanking sequence elements, diverse sequences can function in place of TATA boxes, with specificity of initiation being achieved by the requirement for these nearby elements. Quite likely, the association of hTFIIDτ with different combinations of cellular factors modifies both its binding specificity and the nearby sequence elements with which it can interact to form stable initiation complexes. Thus, different TFIID complexes

Promoter	Oligo. sequence and position rel. to major initiation site	Rel. affinity for hTFIIDτ
Ad-ML	GGGGCTATAAAAGGGGGTGG	1
pSVsKC2	CAGAAGGTATTTAACCAAGT	0.5
hum. DHFR	GCCTGCACAAATAGGGACGA	0.3
rpL32	CCATCATACCCTGCGCGCCG	0.25
SV40-ML	CAGAAGGTACCTAACCAAGT	0.15
TdT	GTACCTATGGGTCTGCTGGT	0.15
IRF-1	AAAGTGTTTAGATTTCTTCGC	0.15
pSVsKA1	CAGAAGGCACCGAACCAAGT	0.05
	-40 -35 -30 -25 -20 -15	

FIG. 4. Summary of relative hTFIIDτ binding affinities for the −30 regions of the TATA-less promoters analyzed in this study. The "sense" strand of each double-stranded oligonucleotide used as competitor is indicated, with its position given relative to the major initiation site. Boxed is the best match of each oligonucleotide in the sense orientation to a weighted sequence matrix optimized to match TATA box elements [determined as described by Bucher (20)]. The percent G+C content of the oligonucleotides is as follows: Ad-ML, 60%; pSVskC2, 35%; human dihydrofolate reductase (hum. DHFR), 55%; rpL32, 70%; SV40-ML, 45%; TdT, 55%; interferon regulatory factor (IRF-1), 35%; and pSVsKA1, 55%. Rel., relative.

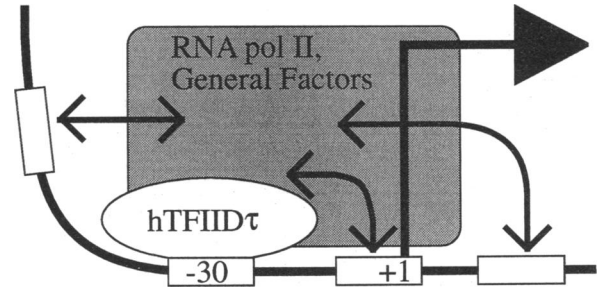

FIG. 5. Context hypothesis for transcription initiation of TATA-less promoters. hTFIIDτ directly binds to a variety of sequences ≈30 bp upstream of the site of initiation. In the right context of cis-acting sequences upstream, downstream, and at the site of initiation (indicated by boxes), other factors interact directly or indirectly with hTFIIDτ (indicated by arrows), and thereby contribute to the formation of a stable initiation complex in a manner qualitatively similar to initiation at TATA box-containing promoters.

probably functionally bind the diverse −30 regions of different promoters.

Lastly, the data presented here and elsewhere (27, 28) indicate that it is not clear what sequences make up a TFIID binding site. For example, the initiation site element of the SV40-MLP contains the sequence TGAAATAA. This sequence more closely matches a statistically derived TATA box-weighted sequence matrix (20) than does the SV40-MLP −30 region sequence. Nevertheless, it is at best only weakly protected from DNase I cleavage by hTFIIDτ under conditions that significantly protect the −30 region (data not shown). Therefore, the affinity of hTFIIDτ for a given sequence must, for now, be determined experimentally. In particular, one must biochemically measure the affinity of TFIID for individual promoters to determine whether any promoters exist that truly lack a TFIID binding site.

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